STUDY OF ECOLOGY AND MOLECULAR CHARACTERIZATION OF LAB MICROFLORA IN SREMSKA SAUSAGE

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Abstract—**This paper describes culture-dependent and-independent methods, to study the ecology of Sremska sausage during fermentation. Sremska sausage represents the Serbian traditional fermented meat product produced using the ingredients such as pork meat, solid fat tissue and spices. At the end of the fermentation, these sausages are stable and safer from a microbiological point of view and possess a longer shelf-life. The microorganisms that are responsible for these transformations are lactic acid bacteria (LAB), coagulase-negative cocci (CNC) and yeasts. The identification of the *Lactobacillus* species that dominate the microflora of fermented sausages is an important step in the development of new starter cultures for meat fermentation. The samples were subjected to microbiological analysis to monitor the dynamic changes of microflora and hygienic quality during storage of the Sremska sausages. Total viable count, LAB, CNC, total coliforms and *Escherichia coli*, fecal streptococci, *Staphylococcus aureus, Salmonella spp.* and *Listeria spp.* were investigated on 0th, 3rd, 4th, 7th, 14th and 28th day of ripening. The LAB isolates from MRS were first checked by Gram stain and catalase reaction. Gram-positive and catalase-negative isolates were then further identified by biochemical (API 50CH) and molecular methods. Total DNA from LAB was extracted from a single colony and DNA V1-V3 region which encodes 16S rRNA was amplified. Positive samples which displayed 697 bp band were purified and sequenced. Results of sequencing underline how *Ln. mesenteroides* was the main bacteria present in the Sremska sausage. Also, two uncultured clones were detected.**

Index Terms—**lactic acid bacteria, fermented sausage, Sremska sausage, sequence analysis.**

I. INTRODUCTION

Fermentation and drying of meat products are some of the oldest technologies used to preserve food for long periods. Highly perishable raw materials, such as milk and meat, undergo relevant changes in the physico - chemical characteristics and at the end of the fermentation, foodstuffs are more stable and safer from a microbiological point of view and possess a longer shelf-life. The microorganisms that are most often responsible for these transformations are lactic acid bacteria (LAB), coagulase-negative cocci (CNC) and yeast [2].

Sremska sausage represents the Serbian traditional fermented meat product produced using the ingredients such as pork meat, solid fat tissue and spices. Good Sremska sausage is of pleasant, aromatic taste with significant smoked and very often sharp smell of chili pepper [5].

In order to protect the traditional aspect of this product, it is essential to understand the microbial ecology during fermentation by studying the dynamic changes that occur [4] and to select autochthonous starter cultures that can be used in the production.

The identification of the *Lactobacillus* species that dominate the microflora of fermented sausages is an important step in the development of new starter cultures for meat fermentation. However, identification of species, particularly within the genus *Lactobacillus*, is not always possible using phenotypic methods such as sugar fermentation profiles or other biochemical/physiological traits [3]. In the last 10 years, a great improvement in the methods for the more rapid and reliable detection and identification of microorganisms has been achieved by the introduction of molecular biology methods based methods.

In this paper, we describe culture-dependent and culture-independent methods, to study the ecology of Sremska sausage during fermentation.

II. MATERIALS AND METHODS

Production of Sremska sausage has been conducted under the controlled industrial conditions. The basic raw material was cooled pork meat of the 1st category and frozen, solid pork fat tissue. The mixtures of nitrate and sodium salt, powdered garlic, powdered red hot chili pepper and dextrose were used as additives. Prepared filling was stuffed into the pork thin casings, 32 mm in diameter. After the leaking and partial drying of the casing, the sausages have been

smoked for 4 days at the temperature of 18°C and relative humidity of 85-90% and the drying and smoking process was continued at the temperature 14-16°C and relative humidity of 75-85% during 24 days. The complete process of Sremska sausage production lasted for 28 days.

The samples for the laboratory examinations were taken on 0^{th}, 3^{rd}, 4^{th}, 7^{th}, 14^{th} and 28^{th} day. Three samples at each step of sampling were collected and used for analysis. The experiment was repeated for three times.

The samples were subjected to microbiological analysis to monitor the dynamic changes during storage of the Sremska sausages and their hygienic quality. In particular, 25 g of each sample was transferred to a sterile stomacher bag and 225 mL of saline-peptone water (8 g/L NaCl, 1g/L bacteriological peptone, Oxoid, UK) was added and mixed for 30 seconds in stomacher. Further decimal dilutions with the same diluents were made and the following analysis were carried out on duplicate agar plates: (a) total viable count on Peptone Agar (8 g/L bacteriological peptone, 15 g/L bacteriological agar, Oxoid) incubated under aerobic conditions for 48-72 h at 30°C; (b) LAB on MRS agar (Oxoid, UK), incubated with a double layer for 48 h at 30°C; (c) CNC on Mannitol Salt Agar (Oxoid, UK) incubated under aerobic conditions for 48 h at 30°C; (d) total coliforms and *Escherichia coli* on VRB agar (Merck, GmbH, Darmstadt, Germany) incubated with a double layer for 24 – 48 h at 37°C; (e) fecal streptococci on Kanamycin Aesculin agar (Oxoid, UK) incubated for 24 – 48 h at 37°C; (f) *Staphylococcus aureus* on Baird Parker medium (Oxoid) with added egg yolk tellurite emulsion (Oxoid) incubated at 37°C for 24 – 48 h; (g) yeast and moulds on Sabouraud-4% Maltose agar (Merck, GmbH, Darmstadt, Germany) incubated for 24 – 48 h at 37°C

Determination of the presence of *Listeria* spp. was performed in accordance with ISO 11290-1:2003 and *Salmonella* spp. according to ISO 6579:2002.

The isolates from MRS were first checked by Gram stain and catalase reaction. Gram-positive and catalase-negative isolates were then further identified by biochemical (API 50CH) and molecular methods.

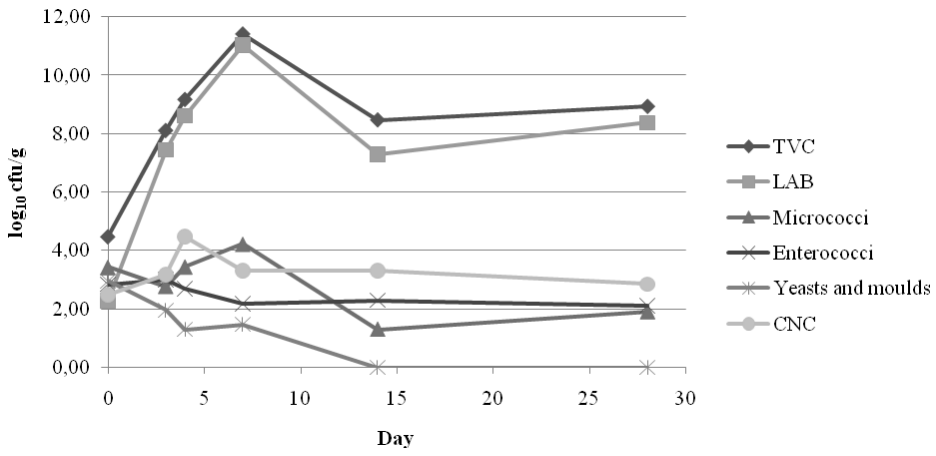
Total DNA from LAB was extracted from a single colony by using the DNeasy Blood and Tissue Kit (Qiagen GmbH, Germany) according to the manufacturer's protocol for Gram-positive bacteria.

PCR was performed in a final volume of 50 µL containing 1xPCR buffer (10× PCR buffer: 500 mM KCl, 100 mM Tris-HCl, 0.8% Nonidet P40), 2.5 mM $MgCl_2$, 200 µM of each dNTP, 2.5 µM of each primer, 1 U of *Taq* polymerase (Fermentas UAB, Lithuania) and 0.1-1 µg of DNA template. The samples were amplified in a DNA thermal cycler (Flexigene, Techne, UK) with primers P1V1 (GCGGCGTGCCTAATACATGC) and P4V3 (ATCTACGCATTTCACCGCTAC), complementary to the V1-V3 region of the 16S rRNA [1], 5 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at 42°C, 2 min at 72°C and the final extension of 5 min at 72°C. PCR products were purified by QIAquick® PCR purification kit (Qiagen GmbH, Germany) and sent for sequencing to *IIT Biotech* (Bielefeld, Germany). The BLAST algorithm was used to determine the most related sequence relatives in the NCBI nucleotide sequence database (http://blast.ncbi.nim.nih.gov).

III. RESULTS AND DISCUSSION

In this paper, we investigated the composition and dynamic of LAB in Sremska sausage at 0^{th}, 3^{rd}, 4^{th}, 7^{th}, 14^{th} and 28^{th} day of the production process. Culture-dependent techniques that have been used for identification of LAB isolates, included morphological (microscopic) analysis, physiological (growth characteristics, simple tests) analysis and biochemical characterization (assimilation and fermentation patterns). Figure 1. shows results obtained by the traditional enumeration of microorganisms of the Sremska sausage during process of ripening.

Figure 1. Microbial counts, determined by plating, during process of ripening



Fifty strains of LAB were isolated from MRS plates during the three fermentations followed and identified by molecular methods, as reported in Table 1.

Table 1: Isolated strains identified by culture-independent methods

Microorganism	Day						TOTAL
	0	***3***	***4***	***7***	***14***	***28***	
Ln. mesenteroides	5 (10%)	3 (6%)	9 (18%)	4 (8%)	6 (12%)	4 (8%)	***31 (62%)***
Pd. pentosaceus	3 (6%)				2 (4%)		***5 (10%)***
W. viridescens		1 (2%)			1 (2%)	1 (2%)	***3 (6%)***
Lc.lactis	1 (2%)	1 (2%)					***2 (4%)***
Lb. sakei		1(2%)				1 (2%)	***2 (4%)***
Lb. plantarum	1 (2%)						***1 (2%)***
Ln. gasicomitatum			1 (2%)				***1 (2%)***
Ln. carnosum		1 (2%)					***1 (2%)***
Lb. curvatus					1 (2%)		***1 (2%)***
Lb. alimentarius						1 (2%)	***1 (2%)***
Uncultured clone	1 (2%)						***1 (2%)***
Uncultured clone			1				***1 (2%)***

The application of molecular methods for the identification of LAB strains highlighted the presence of *Leuconostoc mesenteroides* that was common to all fermentations. Interestingly, at the DNA level an uncultured bacterium were identified as well. This evidence lead to the conclusion that Sremska sausage may contain as-yet-unidentified bacterial species, thus in agreement with previous study [1].

IV. CONCLUSION

The results obtained in this study show that a combined method of cultivation with PCR and subsequent DNA sequencing could successfully identify LAB species from Sremska sausage during the three fermentation. Although several LAB strains, which were previously identified by the culture-dependent method, were not correctly identifed, several additional bacteria were indeed revealed by this culture-independent method.

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